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Introduction of asymmetry in the naturally symmetric restriction endonuclease *Eco*RV to investigate intersubunit communication in the homodimeric protein

(protein–nucleic acids interaction/restriction–modification enzymes/specificity/mechanism of cleavage/cooperativity)

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ABSTRACT **Type II restriction endonucleases are dimers of two identical subunits that together form one binding site for the double-stranded DNA substrate. Cleavage within the palindromic recognition site occurs in the two strands of the duplex in a concerted manner, due to the action of two catalytic centers, one per subunit. To investigate how the two identical subunits of the restriction endonuclease *Eco*RV cooperate in binding and cleaving their substrate, heterodimeric versions of *Eco*RV with different amino acid substitutions in the two subunits were constructed. For this purpose, the *ecor*V gene was fused to the coding region for the glutathione-binding domain of the glutathione *S*-transferase and a His_6-tag, respectively. Upon cotransformation of *Escherichia coli* cells with both gene fusions stable homo- and heterodimers of the *Eco*RV variants are produced, which can be separated and purified to homogeneity by affinity chromatography over Ni-nitrilotriacetic acid and glutathione columns. A steady-state kinetic analysis shows that the activity of a heterodimeric variant with one inactive catalytic center is decreased by 2-fold, demonstrating that the two catalytic centers operate independently from each other. In contrast, heterodimeric variants with a defect in one DNA-binding site have a 30- to 50-fold lower activity, indicating that the two subunits of *Eco*RV cooperate in the recognition of the palindromic DNA sequence. By combining a subunit with an inactive catalytic center with a subunit with a defect in the DNA-binding site, *Eco*RV heterodimers were produced that only nick DNA specifically within the *Eco*RV recognition sequence.**

Type II restriction enzymes are unique enzymes inasmuch as they are symmetric proteins that interact with and cleave their symmetric substrate, a double-stranded DNA with a palindromic recognition sequence, in a symmetric fashion (1, 2). In general, they are composed of two identical subunits that form one binding site for the DNA duplex, as is apparent in the cocrystal structures of *Eco*RI (3), *Eco*RV (4), *Pvu*II (5), and *Bam*HI (6). Under optimal conditions, cleavage of the two strands of the double-stranded DNA occurs in a concerted action of the two catalytic centers, one in each subunit (7, 8). Nevertheless, both catalytic centers are only activated when a cognate substrate is bound. The two identical subunits, therefore, must cooperate in the binding of the substrate, recognition of the cognate site, the activation of the catalytic centers, and possibly (but not necessarily) during catalysis *per se*.

For a study of intersubunit communication underlying this process of cooperation of identical subunits in type II restriction enzymes, we have chosen *Eco*RV, one of the best-studied proteins of this family of enzymes (9). It recognizes the sequence GATATC (10), and in the presence of Mg^{2+} ions, it cleaves DNA specifically in the middle of this site (11). Detailed biochemical studies, which were based on the crystal structure analysis (4), have allowed to identify amino acid residues important for specific DNA binding and catalysis (12, 13), as well to put forward a suggestion as to a likely mechanism of action (14). Of particular importance for DNA recognition are one serine (Ser 183), one threonine (Thr-186), and two asparagine (Asn 185 and Asn 188) residues. For catalysis, one basic (Lys 92) and two acidic (Asp 74 and Asp 90) amino acid residues are of particular importance. When these amino acid residues were substituted by other, even similar, amino acid residues, the resulting *Eco*RV mutants show very little, if any, activity. These *Eco*RV mutants carried the same mutation in both subunits, as conventional site-directed mutagenesis carried out with proteins composed of two identical subunits inevitably leads to protein variants with both subunits carrying the same substitution.

To study intersubunit communication in *Eco*RV, we wanted to introduce amino acid substitutions in only one subunit and study their effects on the other intact or differently substituted subunit. To this end we have produced artificial heterodimers of *Eco*RV by cotransformation of *E. coli* cells with different plasmids, each carrying a different version of the *ecor*V gene, one fused to a gene coding for the glutathione binding domain of the glutathione *S*-transferase (GST), the other to six His codons (15). Upon induction, *E. coli* cells produce homo- and heterodimers that can be separated from each other by affinity chromatography employing glutathione and Ni-nitrilotriacetic acid columns. We have also shown that the isolated homogeneous heterodimer preparation is stable, in the absence and presence of DNA, even at elevated temperature (37°C) (W.W., F.S., and A.P., unpublished data). We describe here the results obtained with several heterodimers, which demonstrate that the two active sites of the *Eco*RV dimer, but not, however, the two parts of the DNA-binding site, function independently of each other. With amino acid substitutions affecting specific DNA binding in one subunit and the active site in the other subunit, an *Eco*RV variant that cleaves the DNA in only one strand of the *Eco*RV recognition site could be produced.

MATERIALS AND METHODS

Expression and Purification of the *Eco*RV Heterodimers. Heterodimers were produced as described (W.W. *et al.*, unpublished data). Briefly, *Escherichia coli* cells were transformed with two plasmids, each carrying the gene for *Eco*RV fused at its 5′-end to the coding region of the glutathione-binding domain of the GST (pGexRVa) or six His codons (pHisRVb), respectively. The expression of the *ecor*V gene,

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Abbreviations: GST, glutathione *S*-transferase; WT, wild-type.

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which in both plasmids is under the control of the inducible p_{tac}-promotor, was performed in *E. coli* strain LK111(λ), bearing the plasmid pLBM4422 (12) that harbors the gene for the *Eco*RV methyltransferase. To produce heterodimers the constructs GST:*Eco*RVa (with mutation a) and His_6:*Eco*RVb (with mutation b) were coexpressed in *E. coli*, leading to the formation of three *Eco*RV dimer species: the two homodimers $(GST:EcoRVa)_2$ and $(His_6:EcoRVb)_2$ and the heterodimer GST:*Eco*RVa/His_6:*Eco*RVb. The $(His_6:EcoRVb)_2$ homodimer was separated by glutathione affinity chromatography, and the $(GST:EcoRVa)_2$ homodimer was separated from the mixture by Ni-nitrilotriacetic acid affinity chromatography. Only the heterodimer GST:*Eco*RVa/His_6:*Eco*RVb binds to both affinity columns, and after elution, it was dialyzed overnight against storage buffer [30 mM potassium phosphate, pH 7.6/0.3 M NaCl/0.5 mM EDTA/0.1 mM 1,4-dithioerythritol/0.01% polyoxyethylene-9-lauryl ether/77% (vol/vol) glycerol] and stored at −20°C. The *Eco*RV heterodimer preparations obtained were homogeneous as determined by SDS/PAGE.

The nomenclature of the heterodimers used throughout the text is demonstrated by the following example: a heterodimer consisting of one subunit with the wild-type (WT) sequence and one subunit with an Asp-to-Ala exchange at position 90 is designated as WT/D90A.

Gel Electrophoretic Mobility Shift Experiments. A 382-bp ^{32}P-labeled DNA fragment (1 nM) with a single *Eco*RV site (16) was incubated with 1, 10, and 50 nM *Eco*RV homo- and heterodimers in binding buffer (50 mM Tris·HCl, pH 7.5/100 mM NaCl/10 mM 2-mercaptoethanol/2 mM spermidine/0.1 mg of bovine serum albumin per ml/5 mM EDTA) for at least 20 min at room temperature. To 10 μl of this binding mixture, 3 μl of gel loading buffer [50% (vol/vol) glycerol/0.25% (wt/vol) xylene cyanol/0.15% (wt/vol) azorubin in binding buffer] was added. Electrophoresis was carried out on 10 × 10 cm 6% polyacrylamide gels at room temperature in TTE buffer (50 mM Tris·taurine, pH 8.0/1.25 mM EDTA). Radioactive bands were detected using an Instant Imager (Canberra Packard, Frankfurt, Germany) or by autoradiography.

Steady-State Cleavage Experiments with Oligodeoxynucleotides. The 20-mer d(GATCGAC**GATATC**GTCGATC) (with the *Eco*RV site in boldface) was synthesized with a MilliGen Cyclone DNA synthesizer. The self-complementary oligodeoxynucleotide was labeled at its 5′-end using T4 polynucleotide kinase (United States Biochemical) and [γ-^{32}P]-ATP (Amersham). Cleavage reactions were performed in 20 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$ using 0.1–1 μM oligodeoxynucleotide and 10 nM–0.5 μM enzyme. After defined time intervals, aliquots were withdrawn and spotted onto DEAE thin-layer plates (Machery and Nagel, Düren, Germany), which were then subjected to homochromatography (17). The detection and quantitation of the separated substrate and product spots was performed using an Instant Imager. The apparent first-order cleavage rate was determined from the initial part of the reaction progress curve. For K_M and k_{cat} determinations, at least four reaction progress curves at four different substrate concentrations were measured for each protein. For WT *Eco*RV, the enzyme concentration was 10 times lower than the lowest substrate concentration, and for the heterodimers, the enzyme concentration was 2–10 times lower than the substrate concentration. The initial velocities were used to determine K_M and k_{cat} values by a least-squares fit to the Michaelis–Menten equation.

Cleavage Experiments with Plasmid DNA and Plasmid DNA Fragments. All cleavage experiments with macromolecular DNA substrates were performed at 37°C in 20 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$ with the supercoiled plasmid pAT153 (18) or the 382-bp DNA fragment mentioned above as substrates containing a unique site for *Eco*RV. Cleavage experiments were performed by diluting the stock solution of WT *Eco*RV to 10 nM and the heterodimers to 500 nM. Aliquots (10 μl) of these dilutions were added to 90 μl of a solution of 350 nM pAT153 in reaction buffer. After defined time intervals, 10-μl aliquots were withdrawn and immediately mixed with 5 μl of stop-mix (0.25 M EDTA/25% sucrose (wt/vol)/1.2% SDS/0.1% bromophenol blue/0.1% xylene cyanol, pH 8.0). Substrates and products were separated by electrophoresis in 1% agarose gels under native conditions in TPE (80 mM Tris·phosphate, pH 8.0/2 mM EDTA) or in 8% polyacrylamide gels under denaturing conditions (7 M urea) in TTE (80 mM Tris·taurine, pH 9.0/2 mM EDTA). Agarose gels were stained with ethidium bromide and analyzed with the Intas gel documentation system (Intas, Göttingen, Germany). Polyacrylamide gels were analyzed using the Instant Imager.

RESULTS

To study the communication between the two identical subunits of the *Eco*RV homodimer, the heterodimers WT/D90A, WT/N188Q, WT/T186S, D90A/N188Q, D90A/T186S, and WT/[D90A,N188Q] were produced. The location of the amino acid substitutions in these heterodimers is shown in Fig. 1. In the first kind of heterodimers, one subunit has the WT sequence, while the other subunit has a mutation in the catalytic center (WT/D90A) or in the DNA recognition loop (WT/N188Q and WT/T186S). In the second kind of heterodimers, two different mutations are present, one in each subunit (D90A/N188Q and D90A/T186S). In the third kind, one subunit has the WT sequence, while the other subunit contains two mutations (WT/[D90A,N188Q]). For reference, a WT enzyme was produced and used in all experiments that carries both tags, one subunit with the GST-tag and the other subunit with the His_6-tag (WT/WT).

To demonstrate the purity and stability of the heterodimeric preparations, a gel electrophoretic mobility shift assay was used. These experiments were carried out in the absence of Mg^{2+}; under these conditions, *Eco*RV, unlike *Eco*RI, *Rsr*I, *Bam*HI, and *Fok*I (19–23), but like *Taq*I, *Cfr*9I, and *Bcg*I (24–26), binds nonspecifically to DNA, resulting in a ladder of multiple band shifts (27). The mobility of the heterodimeric 1:1 *Eco*RV–DNA complex is intermediate between the mobility of the 1:1 complex of the homodimers (Fig. 2). The result of this

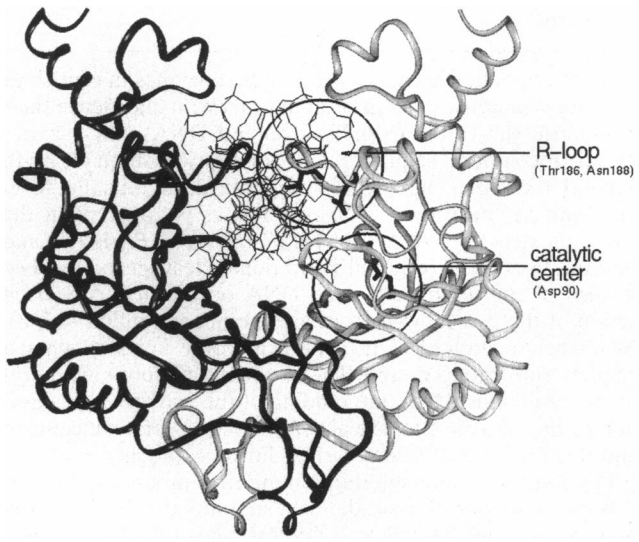

FIG. 1. Structure of the specific *Eco*RV–DNA complex (Brookhaven Protein Data Bank entry no. 1RVB). The enzyme is a dimer of two identical subunits. The DNA recognition loop (R-loop with the side chains of amino acid residues Thr-186 and Asn-188 displayed) and the catalytic center (with the side chain of amino acid residue Asp-90 displayed) are indicated in each subunit.

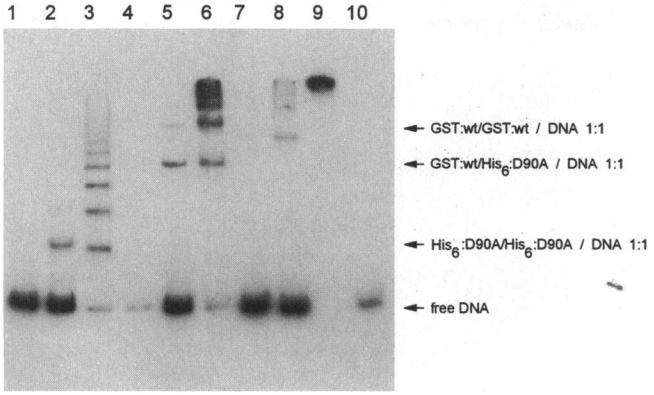

FIG. 2. DNA binding by *Eco*RV homo- and heterodimers. The binding mixture contained 1 nM 382-bp DNA fragment in binding buffer and $(His_6:D90A)_2$ (lanes 1–3), GST:WT/His_6:D90A (lanes 4–6), and $(GST:WT)_2$ (lanes 7–9) at increasing concentrations (lanes 1, 4, and 7, 1 nM; lanes 2, 5, and 8, 10 nM; and lanes 3, 6, and 9, 50 nM). Lane 10 shows the free DNA in the absence of *Eco*RV. Under the conditions of the experiment, *Eco*RV binds nonspecifically to DNA. The free DNA and the first band shift (1:1 complex) are marked.

and similar experiments carried out with the other heterodimer preparations demonstrate that the preparations do not contain detectable amounts of the homodimeric proteins and confirm that the heterodimers do not exchange subunits upon purification and storage. Band shift experiments performed after the heterodimers had been incubated for 1 hr with DNA under cleavage conditions demonstrated that under these conditions the heterodimers also do not recombine (data not shown).

The activities of all heterodimers were determined with a 20-mer oligodeoxyribonucleotide substrate (Fig. 3). The Michaelis–Menten parameters (K_M and k_{cat}) derived from the oligodeoxynucleotide cleavage experiments are given in Table 1.

Compared with WT/WT, the heterodimeric variant WT/D90A has a 2-fold reduced specific phosphodiesterase activity (k_{cat}/K_M). This is exactly what would be expected if one active and one inactive subunit were present, and if the two catalytic centers did not influence each other. In contrast, the specific

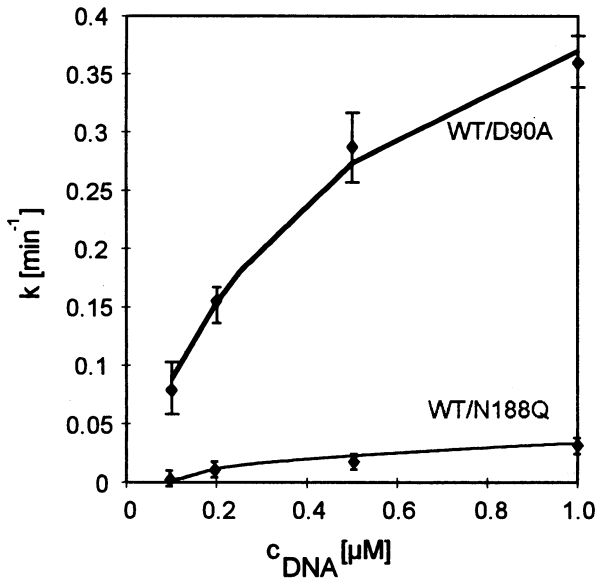

FIG. 3. Steady-state kinetic analysis for the cleavage of the 20-mer oligodeoxynucleotide by the *Eco*RV heterodimers WT/N188Q (lower curve) and WT/D90A (upper curve). Data points with standard deviations were derived from at least four independent measurements.

Table 1. Michaelis–Menten constants for the cleavage of d(GATCGA**CGATATC**GTCGATC) by *Eco*RV homo- and heterodimers

Enzyme	K_M, μM*	k_{cat}, min^{-1}*	k_{cat}/K_M $min^{-1}\cdot mM^{-1}$
WT/WT	0.82	1.85	2.26 (1.0)†
WT/D90A	0.54	0.57	1.05 (0.47)†
WT/N188Q	0.80	0.05	0.06 (0.03)†
WT/T186S	0.72	0.025	0.04 (0.02)†
WT/[D90A,N188Q]	0.57	0.021	0.04 (0.02)†
D90A/N188Q	does not cleave oligonucleotide substrates; polynucleotides are nicked		
D90A/T186S	does not cleave oligonucleotide substrates; polynucleotides are nicked		

Boldface denotes the *Eco*RV site.
*All values are derived from experiments carried out at least four times; they are accurate within at least ±30%.
†Relative values with WT k_{cat}/K_M set to 1.

phosphodiesterase activity of the WT/N188Q and the WT/T186S heterodimers is 30- and 50-fold reduced, respectively, compared with WT *Eco*RV. This result is most easily explained by assuming that in these heterodimers the catalytic function of *both* subunits is severely disturbed by the mutation in only one subunit. The Michaelis–Menten analysis of oligodeoxynucleotide cleavage by these heterodimers show that their reduced catalytic activity is exclusively due to a reduced k_{cat} value. This suggests that it is not so much DNA binding that is affected by the N188Q or T186S mutations in one subunit but rather the coupling of specific binding and catalysis.

To find out to what extent each catalytic center is influenced by a mutation in one DNA recognition loop, we generated the heterodimers D90A/N188Q, D90A/T186S, and WT/[D90A,N188Q]. With the heterodimers D90A/N188Q and D90A/T186S, we could examine within one subunit the dependence of catalysis on the integrity of the R-loop, whereas with the heterodimer WT/[D90A,N188Q], it was possible to investigate the influence of a defect in the recognition loop in one subunit on the catalytic center in the other subunit. The D90A/N188Q and D90A/T186S heterodimers are not able to cleave the 20-mer oligodeoxynucleotide, which demonstrates that with these defects in the DNA recognition loop the catalytic center in the same subunit cannot be activated by this substrate. This result suggests, furthermore, that the residual activities of the WT/N188Q and WT/T186S heterodimers are due to the catalytic activity of the active center in the WT subunit. In agreement with this suggestion is our finding that the WT/[D90A,N188Q] heterodimer behaves like the WT/N188Q heterodimer: it cleaves the 20-mer with a rate reduced by two orders of magnitude compared to WT *Eco*RV.

Cleavage experiments using supercoiled pAT153 DNA as a substrate demonstrate that WT *Eco*RV cleaves both strands of the DNA in a concerted manner, because the supercoiled plasmid is converted to linear DNA without accumulation of a nicked circle as an intermediate (Fig. 4*a*). In contrast, cleavage of pAT153 by the heterodimer WT/D90A is associated with an accumulation of the nicked circle (Fig. 4*b*), because after cleavage of the DNA in one strand, this heterodimer has to dissociate from the DNA. It then binds again to the nicked site in a new orientation, which allows the catalytically active subunit to cleave the other strand. The heterodimeric variants D90A/N188Q and D90A/T186S, which are unable to cleave the 20-mer oligodeoxyribonucleotide *nick* supercoiled pAT153 DNA. Nicking occurs with a 25- and 50-fold reduced rate, respectively, compared with WT *Eco*RV (Fig. 4*c*). The nick is specific (Fig. 5*b*) and occurs with preference in one strand of the DNA (Fig. 5*c*), as shown by digestion of the 382-bp shift fragment with one *Eco*RV site and analysis of the digestion products by electrophoresis on a denaturing gel (Fig. 5). These findings, as well as the fact that

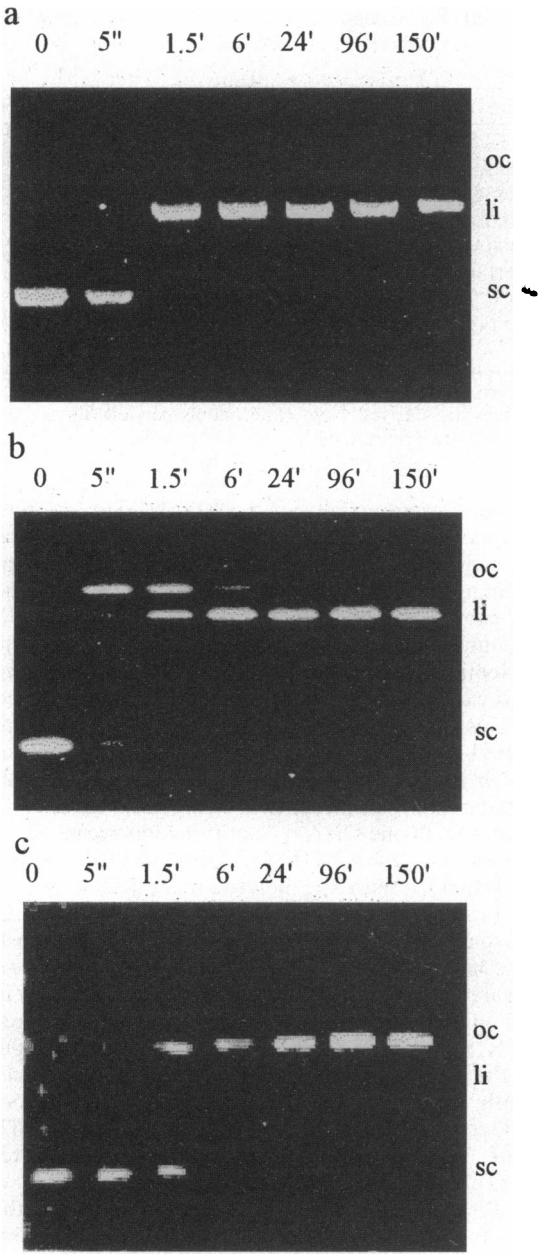

FIG. 4. DNA cleavage by WT *Eco*RV (*a*), the WT/D90A heterodimer (*b*), and the D90A/N188Q heterodimer (*c*). The cleavage reactions were carried out with 350 nM pAT153 DNA (which has a single *Eco*RV site) and 2 nM WT *Eco*RV (*a*), 50 nM WT/D90A (*b*), and 50 nM D90A/N188Q (*c*), respectively. The WT enzyme cleaves both strands in a concerted reaction and the WT/D90A heterodimer cleaves one strand after the other, which leads to the accumulation of the nicked circle. The D90A/N188Q heterodimer only nicks the DNA. sc, supercoiled form of pAT153; oc, nicked circle form of pAT153; and li, linear form of pAT153.

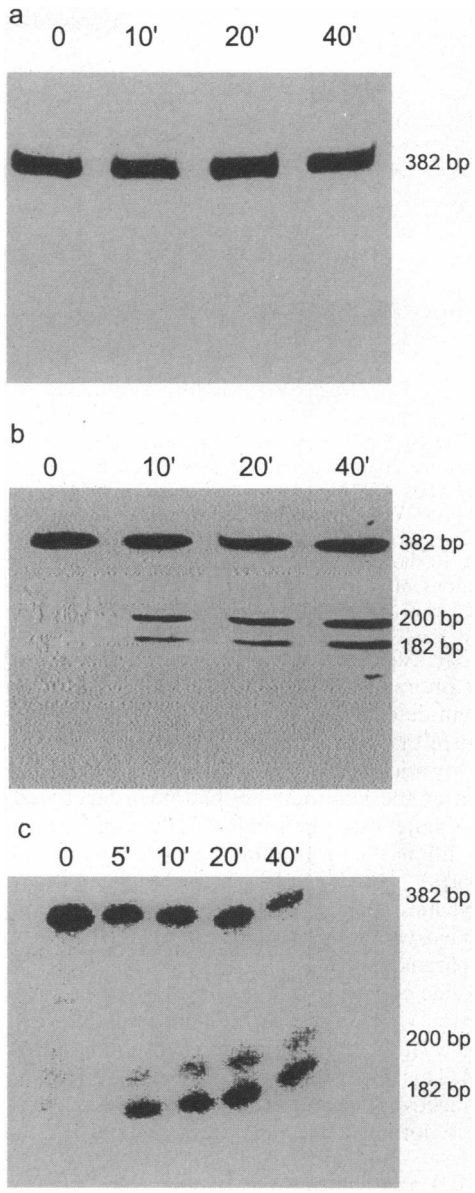

FIG. 5. DNA nicking by the D90A/N188Q heterodimer. The reaction was carried out with 100nM of a uniformly labeled (*a* and *b*) or a 5′-end-labeled (*c*) 382-bp DNA fragment and 50 nM D90A/N188Q. The electrophoretic analysis of the reaction products carried out on native (*a*) and denaturing gels (*b* and *c*), respectively, demonstrate that the fragment is not cleaved (*a*) but nicked specifically at the *Eco*RV site (*b*). Nicking occurs with ≈3-fold preference within the AAGT**GATATC**GGAT strand as compared with the ATCC**GATAT**CACTT stand (*c*). Boldface denotes the *Eco*RV site.

pUC8 DNA, which does not have an *Eco*RV site, was not attacked at all, excludes a contaminant activity of a nonspecific nuclease. It must be emphasized that even after prolonged incubation of pAT153 DNA with D90A/N188Q or D90A/T186S, double-strand cleavage is not observed. To find out whether the inactivity of the 20-mer to function as a substrate for the D90A/N188Q and D90A/T186S heterodimer is due to the particular sequence of the 20-mer oligodeoxyribonucleotide or its length, we have converted the 20-mer oligodeoxyribonucleotide to a polynucleotide by incubation with T4 DNA ligase and ATP. The substrate obtained is attacked by D90A/N188Q and the D90A/T186S heterodimer with a similar rate as plasmid DNA (data not shown), demonstrating that the 20-mer oligodeoxynucleotide is too short to activate the catalytic center of the D90A/N188Q and D90A/T186S heterodimers.

DISCUSSION

To fulfill their biological function, namely to restrict incoming phage DNA, type II restriction endonucleases have to cleave the foreign DNA so that it cannot be easily repaired. This is achieved by a more or less simultaneous cut in both strands of the duplex, which, in contrast to a nick in only one strand, is not effectively resealed by DNA ligase (28). Accordingly, type II restriction enzymes are usually dimers of identical subunits, each of which harbors part of the DNA-binding site and a catalytic center. On the other hand, to avoid nicking of the host

DNA at sites that resemble the recognition site, each catalytic center should only be activated when all sequence specific contacts are formed by both subunits. Hence, the two subunits must cooperate in the recognition process, which includes specific DNA binding and activation of the catalytic centers (29). To study the structural basis of this communication in a prototype type II restriction endonuclease, *Eco*RV, we have carried out a mutational analysis. It was necessary for this purpose to produce mutant variants of *Eco*RV with different amino acid substitutions in the two identical subunits of *Eco*RV. With these artificial heterodimers, it is possible to analyze, for example, the effect of a substitution in the DNA-binding site of only one subunit on the catalytic center of the other subunit.

According to the cocrystal structure analysis of an *Eco*RV–DNA recognition complex (4, 30) and site-directed mutagenesis studies (12, 13), specific recognition is to a large extent due to base-specific contacts originating from the recognition (R)-loop of *Eco*RV (amino acid residues Gly-182–Ser-183–Gly-184–Asn-185–Thr-186–Thr-187–Asn-188), whereas the catalytically important residues are found as part of a sequence motif conserved in many restriction endonucleases (Pro-73–Asp-74... Asp-90–Leu-91–Lys-92; refs. 12, 31, and 32). It has been shown that Ser-183, Asn-185, Thr-186, and Asn-188, as well as Asp-74, Asp-90, and Lys-92 are essential for cleavage; of interest here is the finding that the substitution of Thr-186 by Ser and Asn-188 by Gln led to mutants with no or hardly any detectable activity. Likewise, the exchange of Asp-90 by Ala resulted in a catalytically inactive enzyme, which, however, binds specifically to DNA with high affinity (12, 13). Fig. 1 shows that the R-loop and the catalytic center are not in direct contact with each other, making it obvious that conformational changes are necessary to activate the catalytic machinery upon specific binding. On the other hand, the R-loops of the two subunits contact each other (but only in the specific complex) such that the required cooperation of the two subunits in specific binding can be rationalized by the structure (4, 30).

The steady-state kinetic analysis of the cleavage of an 20-mer oligodeoxyribonucleotide substrate by artificial heterodimers of *Eco*RV presented here demonstrate that the catalytic centers of the two subunits of *Eco*RV do not influence each other, because an inactivating amino acid substitution in one catalytic center, as in the WT/D90A heterodimer, leaves the catalytic center of the other subunit unaffected. In contrast, cleavage experiments with heterodimers carrying amino acid substitutions in the DNA-binding site in one subunit (as in the WT/T186S and WT/N188Q heterodimers) demonstrate that signals originating from specific DNA contacts of one subunit are transmitted to the catalytic centers of both subunits. As a matter of fact, certain amino acid substitutions in the DNA-binding site of one subunit abolish or almost abolish the activity of both catalytic centers. This can be derived from the fact that, for example, the WT/N188Q heterodimer has only a residual activity of 3%. The similar activities of the WT/N188Q and the WT/[D90A,N188Q] heterodimers, as well as the inactivity of the D90A/N188Q heterodimer, suggest that the residual activity of WT/N188Q is due to the catalytic center located in the WT subunit of this heterodimer. It is intriguing to note that the heterodimers that carry an amino acid substitution in the DNA-binding site of one subunit have almost the same K_M value as the homodimeric WT enzyme, but they have a dramatically altered k_{cat} value. This result means that these heterodimeric *Eco*RV mutants are not affected in ground state binding but rather in transition state stabilization, suggesting that the defect in one DNA recognition loop is transmitted to the catalytic centers of the two subunits only during the transition state. By a complementary approach, analyzing the binding of mismatch oligodeoxynucleotides by *Eco*RV, it was also shown that catalysis is much more affected than binding by disturbing the symmetry of the protein–DNA interface (33). On the basis of the cocrystal structure candidate amino acid or nucleotide residues responsible for inter- and intrasubunit communication can be envisaged. Asn-188 interacts via a water molecule with the phosphate to be attacked and with Lys-92 (30), which is likely to be involved in neutralizing the extra negative charge on this phosphate during the transition state (13, 14). This may explain how the catalytic center of one subunit is activated by a contact formed in the DNA-binding site of the same subunit. On the other hand, one could envisage that the physical contact between the two DNA-binding sites (R-loops) allows for a communication between Asn-188 from one subunit and the catalytic center of the other subunit via a conformational change transmitted through the R-loop of the other subunit. Inter- and intrasubunit communication could also be mediated through conformational changes of the phosphodiester backbone of the DNA substrate.

An unexpected outcome of our study has been the generation of heterodimeric *Eco*RV variants that only nick DNA within the *Eco*RV recognition site. Although it had been anticipated that artificial heterodimers of *Eco*RV with one catalytically inactive subunit would cleave the two strands of a DNA duplex in two separated binding events and, therefore, allow for an accumulation of the open circular intermediate, it came as a surprise that heterodimers with an amino acid substitution in the catalytic center of one subunit and another one in the DNA-binding site of the other subunit were unable to perform double-strand cleavage. This finding can be rationalized by arguing that a nicked intermediate cannot productively interact with a heterodimer with a subunit defective in specific DNA binding, because it has freedom of conformational states not accessible to intact double-stranded DNA. By a similar argument it can be explained why oligodeoxynucleotide substrates are not even nicked; when short substrates, which are more flexible than macromolecular substrates because their ends are not constrained, encounter a heterodimer with defects in one DNA recognition loop and in the other catalytic center, they will evade to adopt the conformation required for phosphodiester bond cleavage.

In conclusion, with the methodology that we have developed using artificial heterodimers, one can now specifically address questions regarding inter- and intrasubunit communication in *Eco*RV as well as in other homodimeric enzymes of sufficient stability.

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